REMARKS

Claims 1-7, 9-10, 12, 14-23, 38, 40-61 are amended herein. Claims 26-37 are canceled herein. Claims 8, 13, 24-25, and 39 were previously canceled. New claim 62 is added herein.

Support for the amendment is found, for example, in the specification on page 9, lines 6-12, page 12, lines 23-29, page 25, 2nd and 3rd paragraphs, page 29, lines 14-16, Example 7 beginning on page 56, and in the original claims. Hence no issues of new matter are presented.

Accordingly, upon entry of the amendment, claims 1-7, 9-10, 12, 14-23, 38 and 40-62 will be all of the claims pending in the application.

Entry and consideration of this Amendment are respectfully requested.

Applicants submit the following remarks in addition to the amendments to the claims to address issues raised in the parent application for the Examiner's consideration.

I. Response to Claim Objections

Claim 4 was objected to as an improper multiple dependent claim. Claim 4 is amended herein to refer to depend from claim 3, thereby obviating the objection.

II. Response to Claim Rejections Under 35 U.S.C. § 101

Claims 7, 9, 12, 14, 38 [sic], 40, 41 and 51-61 were rejected under 35 USC § 101 allegedly because the claimed invention is directed to non-statutory subject matter. Specifically, the Examiner stated that the claims are directed to yeast strains that can arise naturally and therefore there is an absence of the "hand of man" in the claimed invention. The Examiner suggested that amending the claims to recite that the claimed yeasts are "isolated" or "recombinant" would be sufficient to overcome the rejection.

The claims in the present application are amended to recite an "isolated" yeast strain, thereby obviating the rejection.

III. Response to Claim Rejections Under 35 USC §112, 1st Paragraph

Claims 7, 9, 10, 12, 14, 38, 40, 41 and 51-61 were rejected under 35 USC § 112, 1st paragraph, for failing to comply with the written description requirement.

Claim 7 is amended herein to recite an industrial isolated yeast strain of the fil phenotype, which is obtainable by the process according to claim 1. Thus, the recited strain is described in terms of the process for making it, thereby obviating the rejection. Claims 9, 10, 12, 14, 38, 40, 41 and 51-61 depend from claim 7 and are adequately described in terms of the process for making the recited products.

IV. Claim Rejections Under 35 USC § 112. 2nd Paragraph

Claims 1-7, 9, 10-, 12, 14-23, 38 and 40-61 were rejected under 35 USC § 112, 2nd paragraph, as allegedly being indefinite.

The claims are amended herein to clarify the claim language and for consistency.

With respect to the Examiner's concerns regarding the term "repeated culture" Applicants respectfully submit that such knowledge is within the skill of the ordinary artisan and in the specification an example "of after 10 successive cultures" is disclosed on page 26 for determining whether the fil phenotype is stable. Thus, when properly read in light of the specification, one of ordinary skill in the art would readily understand the meaning and scope of the claims.

Claim 4 is amended herein to clarify the claim language.

Example 7 is an example provided in the specification of the process according to claim 4. In Example 7, the industrial starting yeast strain is strain S47, which is a polyploid strain. See page 29, line 15. The selected industrial fil mutant yeast strain obtained through mutations of an industrial yeast strain is the mutant AT25 or S47 *fil400*. As indicated in the first lines of Example 7, AT25 carries several mutations with respect to the starting strain.

Segregants of the industrial mutant yeast strain are then obtained. The segregants of the industrial fil mutant yeast strain AT25 are crossed with the laboratory haploid W303-1A, resulting in a first family of polyploids. Thus, it is clear from Example 7 that the term "crossing" refers to "mating".

The polyploids of the first family are evaluated according to tests T6 (glucose consumption of non-frozen cells) and T7 (glucose consumption after freezing). The segregants corresponding to polyploids with improved results (higher glucose consumption) in tests T6 and T7 (with respect to the industrial starting strain and the industrial fil mutant) are selected. See page 58 of the specification. The selected segregants are crossed, or mated with one another, resulting in a second family of polyploids.

The polyploids belonging to the second family that satisfy the selection criteria which are characteristics of the fil phenotype are selected. In Example 7, an example of the method of claim 4, AT251, AT252 and AT254 are the resultant strains.

Thus, one of ordinary skill in the art would readily be able to ascertain the meaning and scope of claim 4 as amended herein.

With respect to the term "industrial strain" and "laboratory yeast strains", Applicants submit that these terms are easily understood when properly read in light of the specification. For example, on page 10, at lines 16 to 17 of the specification, it is disclosed that an industrial yeast strain is a strain used in optimized and competitive industrial production of, for example, baker's yeasts or bread making yeast strains as in the present application. At lines 23-24, it is further disclosed that industrial strains are usually polyploids. Aneuploids and polyploids are mentioned as industrial strains on page 11 line 31.

Laboratory strains are defined in the specification as a model strain which does not have all of the properties necessary to be industrial strains and as a true haploid or a true diploid of *Saccharomyces cerevisiae*. Further, on page 11, lines 27-28 and page 12, line 4, true haploids or diploids are mentioned as laboratory strains. Additionally, industrial yeasts are differentiated from laboratory yeast strains in the specification on page 10, lines 20-24.

Further, the terms "industrial yeast strains" and "laboratory yeast strains" are terms of art that are readily understood by those of ordinary skill in the art. These terms are frequently used in reference manuals and scientific publications, often without additional explanation or detail, since such further detail is not deemed necessary by the skilled artisan. By way of illustration, Applicants submit the following herewith:

Attachment A. Reed et al, Yeast Technology, 2nd Ed., 1991, pages 38-39, wherein laboratory and industrial yeast strains are discussed in the last two paragraphs on page 38;

Attachment B. Tara Young, "High Yield Glutathione Yeasts", at http://www.aussieopportunities.com.au/?page=search_regiostration, a description of a research project downloaded from the internet, which mentions industrial and laboratory strains, and makes a distinction between the tow without further detail; and

Attachment C. Yuji Oda et al, "Principal-Component Analysis of the Characteristics Desirable in Baker's Yeasts", Appl. Environ. Microbiol., June 1989, p. 1495-1499, an article in which it is clearly indicated in the first paragraph that (Saccharomyces) yeast strains are generally grouped into two types: laboratory strains and industrial strains.

Thus, in view of the above, the terms "industrial strains" and "laboratory strains" are terms of art, which are recognized as such and readily understood by those of ordinary skill in the art.

In regard to the term "a bad smell or a bad or abnormal taste", Applicants respectfully submit that such is a term of art within the common knowledge of the skilled artisan. As indicated in the above-mentioned Yeast Technology reference on page 5 (Attachment D):

> Strain selection and strain improvement in the baker's yeast industry are aimed at achieving two important goals: achieving superior bake activities and providing a low-cost baker's yeast without affecting [bakery]product quality (emphasis added).

Thus, it is shown that it is part of the common knowledge of one of ordinary skill in the art of yeast strain improvement that, when developing new industrial yeast strains for use in bread-making, the person skilled in the art must check two distinct aspects before selecting a given strain for industrial use:

1) improved bake activities and low production costs of the yeast strain (in the present case in particular: resistance to stress, fermenting capacity and growth (see the selection criteria in amended claim 1, which are characteristics of a fil phenotype)); and

2) no detrimental effect on the bakery product quality.

According to the first aspect above, as the new yeast strain has improved bake activities; i.e., improved fermentation or leavening activity in the dough, the second aspect of <u>unaffected bakery product quality</u>, essentially refers to the organoleptic properties (taste and smell) of the bakery product.

Therefore, it is clear that the characteristic of the new yeast strain of not leading to a bad smell or a bad or abnormal taste in bread is a common aspect of yeast strain improvement processes as practiced by the person skilled in the art.

In regard to the concern with the perceived relative and subjective nature of the phrase "a bad smell or a bad or abnormal taste", Applicants submit that sufficient guidance is provided in the specification as mentioned on page 9, lines 26 to 29, page 24, lines 19 to 23 and page 26, lines 19 to 22.

The most appropriate adjectives for qualifying the objectionable tastes and/or smells, which, if detected in bread, lead to the rejection of the corresponding yeast strain are:

1. abnormal, and, in particular,

2. off (meaning: in bad condition, wrong, abnormal; odd; spec. . . . (b) of food) stale, sour, beginning to decay, contaminated". The New Shorter Oxford English Dictionary, 1993 Ed.

These terms are not descriptive of personal preferences and dislikes, but of a matter of fact. They refer to characteristics or properties <u>that are alien to the normal product</u>. For example, the question whether milk is off, is a question of fact and not a question of personal preference or dislike for sour milk.

In the present context, in the course of the process according to the claimed invention, yeasts strains may be created that lead to an abnormal or off taste or smell in bread and must be discarded. The occurrence of abnormal or off taste and/or smell in bread is due to the production by the yeast affected by the mutation(s) of one or more secondary metabolites in the bread dough giving rise to said abnormal or off taste and/or smell in the bread.

A taste or smell is "abnormal" or "off" if the consumers, as a collective entity, would consider said taste or smell to be abnormal, i.e., alien to the taste or smell of the same type of bread when produced with an industrial baker's yeast strain. This does not necessarily mean that, when considered outside the context of bread-making, the smell or taste must necessarily be considered unpleasant.

Likewise, the person skilled in the art knows the smell and taste profiles of bread types and is able to detect an abnormal or off taste in bread products produced with a specific yeast strain and which is alien to the type of bread product.

The taste and smell criteria therefore does not distinguish between good and bad smells or between good or bad tastes, but instead distinguished between the tastes and smells of a bread product and those tastes and smells that are alien to the bread product.

V. Conclusion

In view of the above, reconsideration and allowance of this application are now believed to be in order, and such actions are hereby solicited. If any points remain in issue which the Examiner feels may be best resolved through a personal or telephone interview, the Examiner is kindly requested to contact the undersigned at the telephone number listed below.

Respectfully submitted,

Jennifer M. Hayes
Registration No. 40,641

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Date: May 28, 2004

(1935) of the Carlsberg Laboratory led to the discovery of the haploid and the diploid phases of the yeast life cycle. These studies made it possible to elucidate the sexual cycle of S. *cerevisiae*. Winge and Laustsen (1938) later demonstrated the technique of achieving interspecific hybrids in yeast that eventually gave birth to the use of S. *cerevisiae* for genetic studies and breeding programs. These studies together with the discovery by Lindegren and his co-workers that yeasts are heterozygous for the mating-type allele marked the beginning of systematic approaches to strain development. Deliberate selection of heterothallic haploid yeast cultures that not only mate and form diploids but also produce spores with high viability resulted in the development of most of the laboratory strains that are widely used in research today.

Genealogy of commonly used laboratory strains is provided by Mortimer and Johnston (1986). Industrial strains differ significantly from laboratory strains. They sporulate very poorly, and when they sporulate they rarely produce tetrads. The spores are generally not viable under conditions generally recognized as conducive to spore germination. However, a sudden burst of knowledge during the last few decades in yeast genetics and molecular biology has led to the application of these principles developed with laboratory strains to the genetic manipulation of industrial strains.

LIFE CYCLE

Most members of the genus *Saccharomyces* exist either in haploid (single set of chromosomes) or diploid (two sets of chromosomes) phases. In the life cycle of yeast, these two phases alternate with each other, although under special circumstances this process may be interrupted by the formation of triploid or tetraploid strains.

Industrial strains are usually diploid, triploid, polyploid, or in most instances aneuploid where the sexual cycle is rarely present. Propagation of yeast cells takes place vegetatively by a process termed *budding*. In this process, the nucleus divides into two by a mitotic division, resulting in the same number of chromosomes in the daughter cells as in the parent cell. It is normal for vegetative cells of industrial strains to propagate by budding as long as the conditions are not conducive for sporulation.

Many laboratory strains that can be maintained in their stable haploid form for many generations are called heterothallic. These haploid cells exist as one of two mating types, designated "a" or "alpha" cells, which are able to fuse with one another to form a diploid cell type. Such conjugation or mating occurs only between haploids of opposite mating types. The diploids containing the a/alpha cell type are unable to mate. Unlike the haploid strains, they can, however, undergo meiosis or reduction division

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ATTACHMENT A

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and sporulate when starved for nitrogen or easily assimilable carbon. Under these conditions, which are conducive for sporulation, each diploid cell produces an ascus containing four spores, two of the mating type "a" and the other two of the mating type "alpha" showing a typical mendelian genetic segregation. Following germination, each haploid spore gives rise to "a" or the "alpha" cell type that is the stable form of the heterothallic species (Fig. 2-1).

Among certain cell populations where the entire culture is derived from a single spore, cell fusion and diploid formation can still occur and such strains of yeast are termed homothallic. These strains have an HO gene on the left arm of chromosome IV capable of bringing about a switch in the mating type either from alpha to a or a to alpha cell types, resulting in a population of mixed mating types with high frequency of switching after the initial budding cycle. Heterothallic species, on the contrary, have a nonfunctional "ho" allele. The mating reaction between a and alpha cells either in the heterothallic or homothallic strains is initiated by a cell agglutination reaction involving complementary oligopeptide pheromones excreted into the environment by each haploid cell type. Haploids of alpha mating type produce the sex factor called alpha factor that can initiate changes only on the a cells. Likewise, haploids of a mating type produce a different pheromone called a factor that can act only on the alpha cells. Diploid (a/alpha) cells neither produce nor respond to pheromones (Sprague, Blair, and Thorner 1983).

The mating type of haploid strains of yeast is determined by the genetic expression of the MAT locus located on the right arm of the chromosome III in close proximity to the centromere. Two other loci known as HML and HMR are found to the left and right of the MAT locus controlling the conversion from MAT_a to MAT_α or vice versa. The HML_α and the HMR_a loci are storage copies of mating-type information for alpha and a factors that are responsible for the switch of the mating type at the MAT locus.

The MAT_a and MAT_α DNA segments, although to a great extent identical, have shown limited sequence homology within a narrow region flanked by X segment on the left and Z segment on the right as shown in Figure 2-2. This region, unique to MAT_a, is termed Ya, made up of 642 base pairs (bps). The corresponding segment for MAT_α is 747 bps long and has been designated as the alpha region. The genetic segments Y_α and Y_a found within HML and HMR are essentially the alpha and the a genes respectively, identical to those found in the MAT locus but generally silent. It is thus clear that the alpha gene is longer than a gene by about 10^2 bps. Consequently, in homothallic strains, a switch in mating type from a to alpha results in a change from a shorter to a longer segment at the MAT locus. During the switch, genetic material associated with HML_α brings about the mating type interconversion from MAT_a to MAT_α and the

YEAST TECHNOLOGY

Second Edition

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Universal Foods Corporation
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An avi Book
Published by Van Nostrand Reinhold
New York

To our wives, Helen (in memoriam) and Swarna

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NOSTRAND REINHOLD BOOK

Research & Development

HIGH YIELD GLUTATHIONE YEASTS

PRODUCT/SERVICE DESCRIPTION

This research has identified yeast strains and fermentation conditions that result in overproduction of glutathione, particularly extracellular glutathione. The research was based on Saccharomyces cerevisiae, however these findings are relevant to increasing glutathione production using other yeasts. Modification of industrial yeast strains to increase their glutathione production could be undertaken based on this patented technology and the inventors' extensive understanding of the cellular processes that are crucial for controlling glutathione production.

TECHNOLOGY

This research identifies yeast mutations and growth conditions resulting in increased release of glutathione into the culture medium. Example results in laboratory strains with a single mutation include extracellular glutathione production of 40 mg/L and intracellular glutathione production of 15 mg/L (compared to the parental strain grown under identical conditions of <1 mg/L and 7.5 mg/L, respectively). Total culture glutathione (intra- and extracellular glutathione) is increased by at least three-fold with single mutants relative to the parental strain. Production may be further increased by using multiple appropriately selected mutations, batch-fed culture, and industrial strains.

PROJECT SPONSOR

University of NSW - Unisearch

PROJECT OPPORTUNITY SUMMARY

The enhanced levels of glutathione production described previously were obtained using laboratory strains in simple batch culture. As mentioned, it is envisaged that the use of continuous fed-batch cultures under optimised growth conditions, in conjunction with cells harbouring multiple appropriately selected mutations, will increase production further. The team is interested in collaborating with industry to scale-up the use of strains identified for commercial production of glutathione or to develop their current research to increase glutathione production by currently used industrial strains:

Unisearch is seeking a commercial partner or partners with the relevant expertise and market access to develop and commercialise the technology. Unisearch can offer worldwide rights to develop, market and sell products based on the technology.

PROJECT STAGE

Development stage

(1) PROJECT NEED

Strategic Alliance Partner

(2) PROJECT NEED

ProjectManagerExecutive

ATTACHMENT B

PROJECT LOCATION

NSW

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Principal-Component Analysis of the Characteristics Desirable in Baker's Yeasts

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Twenty-seven properties considered to be required for good bakery products were examined in 56 industrial and 2 laboratory yeast strains. The data obtained were applied to principal-component analysis, one of the multivariate statistical analyses. The first and second principal components together were extracted, and these accounted for 77.7% of the variance. The first principal component was interpreted as the glycolytic activity of yeast in dough, and the second one was interpreted as the balance of leavening abilities in sweet and flour doughs from the factor loadings. The scattergram on the two principal components was effective in grouping the 58 yeast strains used.

Saccharomyces strains are generally grouped into two types: laboratory strains, which are experimental microorganisms in fundamental biology and which have contributed to recent advances in molecular biology (3), and industrial strains, which are widely used in baking, brewing, distilling, and wine-making processes (10). These industrial yeasts contain both common and distinct characteristics; those for brewing and distilling are similar but different from those for wine making or baking (16). These characteristics consist of many complex properties. As for baker's yeasts, Burrow (5) stated that the following properties were necessary for a fast dough fermentation: (i) high potential glycolytic activity; (ii) ability to adapt rapidly to changing substrates; (iii) high invertase activity; (iv) high potential maltose fermentation; and (v) ability to grow and synthesize enzymes and coenzymes under anaerobic conditions. Osmotic stability in the presence of high sugar concentrations is also desirable in Japan, since sweet goods are favored much more than in North America and Europe. To construct a new, improved yeast for baking, we should first evaluate the properties mentioned above simultaneously and quantitatively.

Multivariate statistical analyses, which are widely used in pure and applied sciences, include techniques such as principal-component analysis, cluster analysis, discriminant analysis, and regression analysis (6). Cluster analysis is a common practice in the numerical taxonomy of bacteria, actinomycetes, and yeasts (7). The group of microorganisms under study is subjected to a wide variety of tests, and the similarity of characteristics between pairs of microorganisms is expressed numerically (15). Microorganisms with a high percentage of similarity are arranged together to form taxonomic groups or clusters in a tree-like diagram or dendrogram (15). Principal-component analysis, an ordination method, has a firm mathematical basis compared with that used in cluster analysis (4). This method can condense all of the variables, with a minimum mathematical loss of information, into two or three principal components which may be used as axes on which the data can be plotted and visualized structurally (13). Furthermore, this technique enables research of the underlying relationships among variables and interpretation of the classification achieved in terms of these variables (13).

From the above-mentioned reasons, principal-component analysis seems to be a powerful technique for investigating the characteristics of industrial yeasts. In the present paper we subjected the yeast properties considered to be required for good bakery products to principal-component analysis and observed the differences between baker's yeasts, laboratory yeasts, and other industrial yeasts.

MATERIALS AND METHODS

Abbreviations. The following abbreviations are used: PC, principal component; DF (0), dough fermentation, gas production rate from dough without the addition of sugar; DFg and DFs, gas production rates from doughs containing glucose and sucrose, respectively; LFm, liquid fermentation, gas production rate from aqueous medium containing maltose and a small amount of glucose; LFg and LFs, gas production rates from aqueous media containing glucose and sucrose, respectively. The numbers in brackets following DF and LF represent the amounts (percentages) of sugars contained in doughs and aqueous media.

Organism. The types and numbers of *Saccharomyces cerevisiae* strains used were as follows: baking (Japan), 8; baking (North America and Europe), 15; wine, 10; sáke, 5; brewing, 9; shochu, 3; whiskey, 3; alcohol, 3; and laboratory, 2. The organisms were maintained in yeast extract-malt extract agar slants (18).

Cultures. Yeast cells were grown in 30 ml of YPD medium, which contained 1% yeast extract, 2% peptone, and 2% glucose, in a 300-ml Erlenmeyer flask. The broth of the YPD medium was inoculated into 270 ml of medium containing 3% sugar (as cane molasses), 0.193% urea, and 0.046% KH_2PO_4 in a 2-liter baffled Erlenmeyer flask. Both of the cultures were incubated for 24 h at 28°C on a rotary shaker (180 rpm). Cultured cells were harvested, washed twice with distilled water, and placed on a porous plate (Nippon Kagaku Togyo Co., Osaka, Japan) for 5 min to adjust the moisture to about 67%. The cells were resuspended in distilled water (66 mg of dry cells per ml) for the following experiments.

Gas-producing activity of yeasts. The leavening and liquid fermentative abilities of the yeast cells were determined at 30°C for 2 h as the volume of evolved gas from doughs and aqueous solutions, respectively (Table 1). The ingredients of the dough (10 g of flour, 5.5 ml of sugar solution [Table 1], and 1.0 ml of yeast suspension) were kept at 30°C and mixed quickly by hand for 1 min after the addition of yeast suspension. Doughs containing from 5 to 30% sucrose (based

* Corresponding author.

ATTACHMENT C

TABLE 1. Amounts of sugar in doughs and liquid fermentation media

Dough		Liquid fermentation medium	
Designation	Sugar content (g/5.5 ml[a])	Designation	Sugar content (g/10 ml[b])
DF[0]	None	LFm[5]	0.69, Maltose, + 0.035, glucose
DFs[5]	0.5	LFs[5]	0.69
DFs[10]	1.0	LFs[10]	1.38
DFs[15]	1.5	LFs[20]	2.76
DFs[20]	2.0	LFs[30]	4.14
DFs[25]	2.5	LFs[40]	5.52
DFs[30]	3.0	LFg[5]	0.69
DFg[5]	0.5	LFg[10]	1.38
DFg[10]	1.0	LFg[20]	2.76
DFg[15]	1.5	LFg[30]	4.14
DFg[20]	2.0		
DFg[25]	2.5		

[a] Distilled water.
[b] Medium (see text).

on the weight of flour) were prepared to correspond to various white bread and sweet goods formulations. The leavening ability of dough containing glucose was assessed to estimate the fermenting activity irrespective of extracellular invertase. With a similar purpose in mind, liquid fermentative abilities were determined by the method of Hino et al. (9) with a mixture of 2.0 ml of yeast suspension and 10 ml of medium containing, per liter, 10 mM phosphate buffer (pH 5.6), 4.6 mg of thiamine hydrochloride, 4.6 mg of pyridoxine hydrochloride, 46 mg of nicotinic acid, 2.29 g of $MgSO_4 \cdot 7H_2O$, 2.86 g of $(NH_4)_2SO_4$, 5.71 g of urea, and a defined amount of sugar (Table 1). Liquid medium containing maltose corresponded to dough without the addition of sugar and in which endogenous maltose was the major sugar.

Enzyme assays. The reaction mixture for determining extracellular invertase contained 100 mM acetate buffer (pH 4.5), 5% (wt/vol) sucrose, and the appropriate amount of intact cells in a total volume of 0.5 ml. After incubation at 30°C for 3 min, the reaction was stopped by the addition of 3,5-dinitrosalicylic acid reagent, and the reducing sugars produced were determined (2).

α-Glucosidase of yeast cells was induced in the aqueous medium used for maltose fermentation, LFm[5] (Table 1). Samples (2 ml) were removed from the medium after 0, 1, 2, 3, and 4 h of incubation. Washed cells were permeabilized by freezing and thawing (11) and used for the assay of α-glucosidase. The activity was determined at 30°C as the hydrolysis of *p*-nitrophenyl-α-D-glucopyranoside by the method of Operti et al. (11). Specific activities of both enzymes were expressed as nanomoles per minute per milligram of cells.

PC analysis. The PC (Z_i) was computed from the following equation (1): $Z_i = a_{i1}x_1 + a_{i2}x_2 + \ldots + a_{ik}x_k + \ldots + a_{im}x_m$, where x_k refers to the k^{th} value of the datum obtained, and the variance in Z_i ($i = 1, 2, 3 \ldots p$) and a_{ik} ($i = 1, 2 \ldots p$, $k = 1, 2 \ldots m$) refers to the eigen value and the eigen vector, respectively. First, a_{1k} values are computed on the basis of a correlation matrix among the data obtained to maximize the variance in Z_1 among all PCs. Then, a_{2k} is calculated to maximize the variance in Z_2 except for Z_1. The computation can be continued until $(m - 1)^{th}$ PC is calculated, but this analysis is usually stopped before the eigen value becomes less than 1.0. Factor loading refers to the coefficient of correlation between x_i and Z_i. Calculation was conducted by the multivariate analysis program (IBC Co. Ltd., Miyazaki, Japan) with the NEC personal computer PC9801.

RESULTS AND DISCUSSION

Twenty-seven properties considered to be required for good bakery products, such as gas-producing activities, enzyme activities, and cell yield, were assayed in 58 yeast strains. Since a good practice in PC analysis is to ensure that the number of variables is less than 25% of the samples (6), the data measured were limited by the following procedure. Four groups of gas-producing activities (DFs, DFg, LFs, and LFg; Table 1) and α-glucosidase (at 0, 1, 2, 3, and 4 h) were subjected to PC analysis. The eigen values of the first PCs extracted from DFg, LFs, LFg, and α-glucosidase surpassed 1.0 and explained 79.4% of the variance in DFg, 75.2% of that in LFs, 88.7% of that in LFg, and 93.1% of that in α-glucosidase. The eigen values of the first and second PCs extracted from DFs surpassed 1.0 and explained 96.7% of the variance. Thus, DFs[15], DFs[30], DFg[15], LFs[20], LFg[20], and α-glucosidase (2 h) were selected as the DFs, DFg, LFs, LFg, and α-glucosidase variables with the highest factor loading for the respective PCs. The correlation coefficient matrix was calculated for these selected variables (Table 2). Significant correlations were observed among gas-producing activities, except for between DFs[30] and LFg[20] and between DFs[30] and LFs[20]. The negative correlation observed between invertase activity and DFs[30] supports the results of a previous report (14). A higher extracellular invertase activity might reduce the fermentative ability of yeast cells, since the hydrolysis of sucrose into glucose and fructose doubles the osmotic pressure around the cells (14). In dough without sugar yeast fermentation depends mainly on maltose, which is derived from the starch in the flour by the action of endogenous β-amylase (11). The leavening ability of the flour dough was expected to correlate closely with both LFm and α-glucosidase, but there have been controversial findings (8). In the present experiment DF[0], LFm[5], and α-glucosidase activity correlated significantly with each other.

The PCs of which the eigen values surpassed 1.0 were the first and second ones, and these PCs accounted for 77.7% of the variance of the data. The factor loadings for each of the 10 variables for the first and second PCs were plotted to investigate the significance of each PC (Fig. 1). The first PC, with which all of the yeast activities correlated except for DFs[30], could be interpreted as the glycolytic activity of

TABLE 2. Properties applied to PC analysis

Parameter	DFs[15] (ml/2 h)	DFs[30] (ml/2 h)	DFg[15] (ml/2 h)	DF[0] (ml/2 h)	LFs[20] (ml/2 h)	LFg[15] (ml/2 h)	LFm[5] (ml/2 h)	α-Glucosidase (nmol/min per mg of cells)	Invertase (nmol/min per mg of cells)	Cell yield (g [dry matter])
Avg	30.2	3.3	21.6	32.7	29.6	25.5	15.6	226	6,365	2.53
Minimum	8.0	0	3.5	6.0	6.5	5.0	0	0.8	254	1.23
Maximum	45.1	10.1	32.5	53.0	53.0	47.0	50.5	773	31,200	3.03

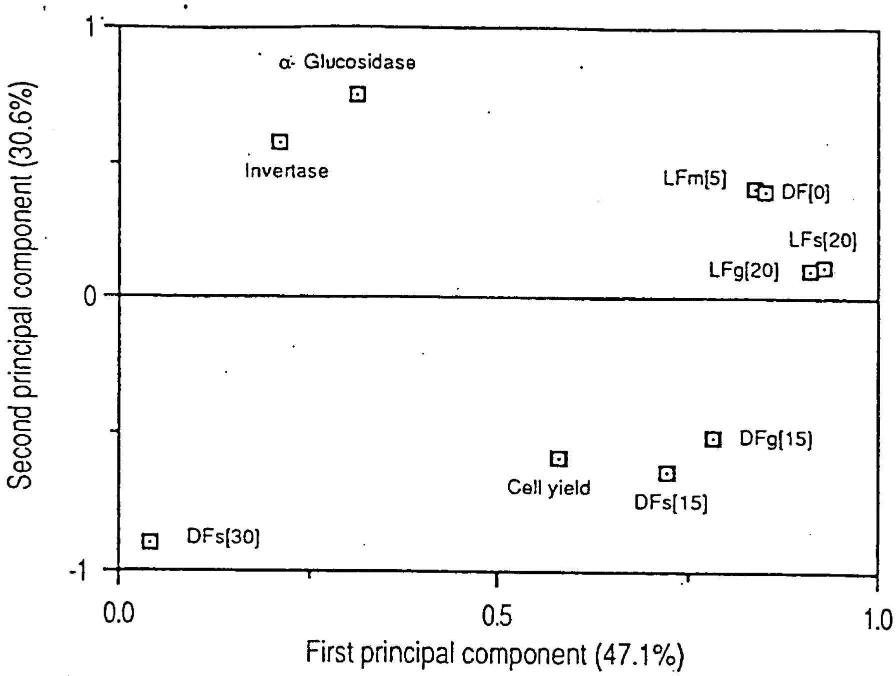

FIG. 1. Scattergram of factor loadings for the first and second PCs.

yeast in dough. DFs[30] and α-glucosidase correlated negatively and positively with the second PC, respectively, suggesting that this PC is related to the balance of leavening abilities in sweet and flour doughs.

The scattergram of 58 yeast strains for the first and second PCs is shown in Fig. 2. The first and second PCs accounted for 47.1 and 30.6% of the variance, respectively, and were effective in grouping the 58 yeast strains. When PC analysis was conducted on all the data except those for α-glucosidase, invertase, and cell yield, some groups overlapped and could not be distinguished from each other (data not shown). The leavening abilities of doughs containing sucrose and the α-glucosidase activities of four representative strains (FSC6001, a Japanese baking strain [no. 1 in Fig. 2]; FSC6014, a European baking strain [no. 12]; BK1111, a brewing strain [no. 43]; and X2180-1A, a laboratory strain [no. 57]) are shown in Fig. 3 and 4.

All of the baker's yeasts were found on the right half of the

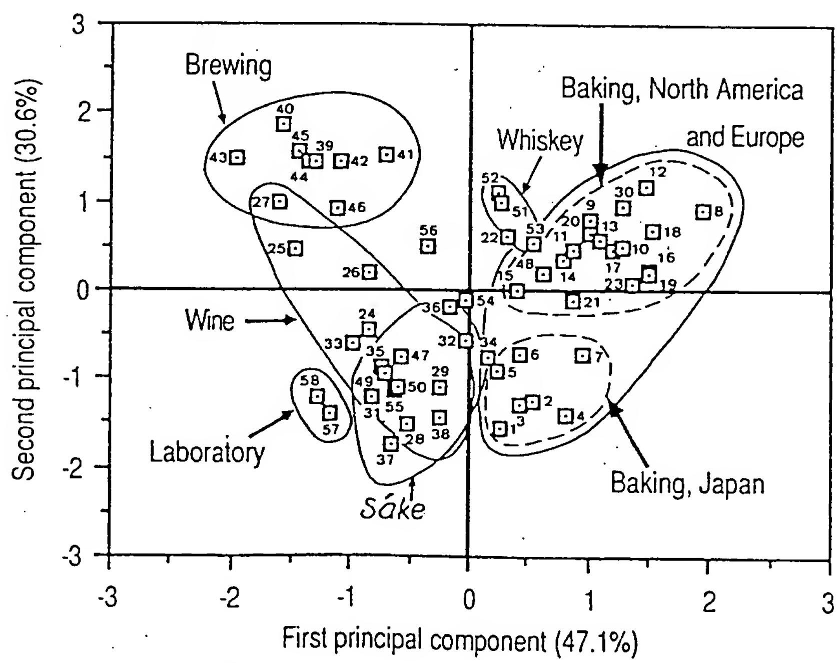

FIG. 2. Scattergram of 58 yeast strains for the first and second PCs. The types of *S. cerevisiae* strains were as follows: 1 to 8, Japanese baker's yeasts; 9 to 23, North American-European baker's yeasts; 24 to 33, wine yeasts; 34 to 38, sǎke yeasts; 39 to 47, brewer's yeasts; 48 to 50, shochu yeasts; 51 to 53, whiskey yeasts; 54 to 56, alcohol yeasts; and 57 and 58, laboratory yeasts.

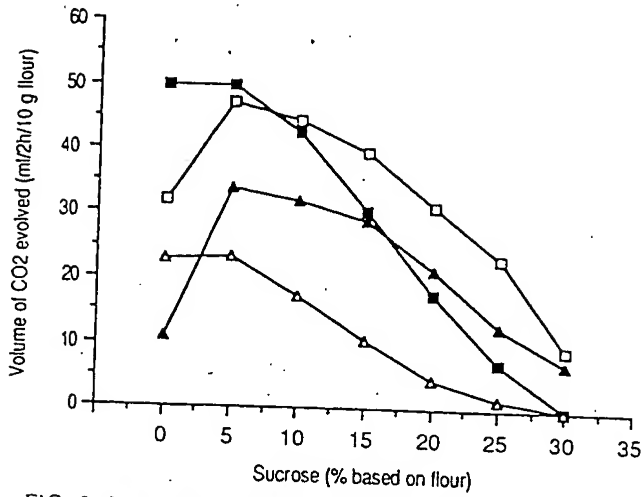

FIG. 3. Leavening abilities of doughs containing sucrose. Symbols: □, FSC6001, a Japanese baking strain; ■, FSC6014, a European baking strain; △, BK1111, a brewing strain; ▲, X2180-1A, a laboratory strain.

scattergram. These positions indicated that baker's yeasts indeed had higher glycolytic activities in dough than did the other industrial yeasts. The separation of Japanese and North American-European baker's yeasts into subgroups along the second axis reflected that accepted dough formulas are different in these countries (17). The exceptional position of FSC6224, a Japanese baking strain (no. 8 in Fig. 2) was reasonable, since the compressed form of this strain is exclusively used for dough containing lower amounts of sugar in Japan.

Brewer's yeasts were clearly differentiated from baker's yeasts. The former organisms have been highly adapted to maltose fermentation but possess poor leavening ability. Since the early records of the history of fermentation showed that foam from beer fermentation was used to raise dough, baker's and brewer's yeasts may have originated from the same ancestral strains (12). Unintentional selection in bread making and brewing over a long period could have caused the differences in these two yeasts. Although the classification of microorganisms by cluster analysis is now

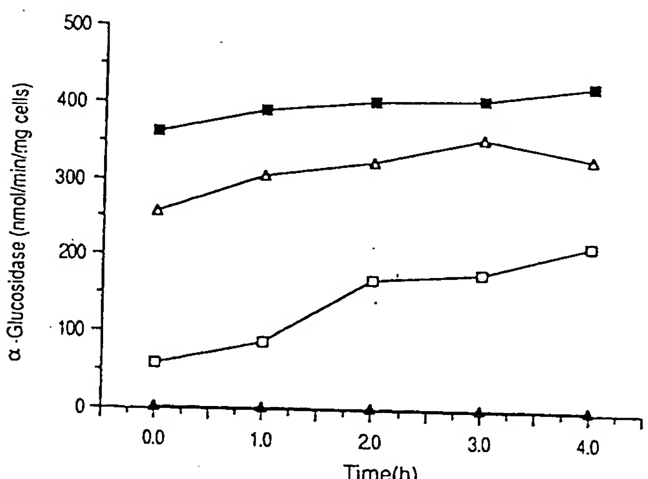

FIG. 4. Induction of α-glucosidase by exogenous maltose. Symbols are the same as in Fig. 3.

common practice, there have been few reports on industrial yeasts except for brewer's yeasts (4). A total of 235 strains of brewer's yeasts from the National Collection of Yeast Cultures (Norwich, United Kingdom) were divided into five groups, each of which consisted of two subgroups determined by principal-coordinate analysis as an alternative to PC analysis on the basis of the data on brewing properties (4).

Two laboratory yeasts were mapped far from baker's yeasts, clearly indicating that these strains could not be used for baking. Whiskey yeasts were placed in the middle of brewer's and North American-European baker's yeasts. Wine, sáke, shochu, and alcohol yeasts overlapped in the map. Brewing and whiskey yeasts, which fermented in the mash containing maltose as a main sugar, seemed to be separated from sáke, shochu, and wine yeasts, which fermented in moromi mash or must containing glucose as a main sugar.

On the basis of PC analysis, we were able to visualize the differences between baker's yeasts, laboratory yeasts, and other industrial yeasts. We plan to search for and evaluate suitable isolates or constructed strains on the scattergram obtained from this analysis.

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imaginative gene manipulations can be attempted, particularly with regard to the genetic makeup of their specific characters and regulations.

Some traits have already been introduced into baker's and brewer's yeasts to improve the usefulness of these strains in their respective applications. These developments are described briefly in the following sections.

BAKER'S YEAST

Strain selection and strain improvement in the baker's yeast industry are aimed at achieving two important goals: achieving superior bake activities and producing a low-cost baker's yeast product without affecting product quality. Under the first category possible targets that are likely to receive consideration in a strain improvement program are rapid maltose fermenting ability (lean dough yeast), improved osmotolerance (sweet dough yeast), rapid fermentation kinetics, and better freeze/thaw tolerance. Baker's yeast production can also be made more cost-effective by using novel strains that have rapid melibiose-utilizing ability (when beet molasses is used as a substrate) or rapid lactose-utilizing ability (when whey is used as a substrate).

Improvement of Fermentation Characteristics

Rapid Maltose Adaptation

Types of baker's yeast have changed in recent years to accommodate extensive marketing strategies, and the traditionally used strains have now become inadequate to satisfy the baker's complex demands. The baking industry characterizes dough systems by the level of sugar that is included in the respective formulations. Lean dough formulations often include flour, water, shortening, salt, and yeast with no added sugar. In this dough system, the main source of fermentable sugar is produced when the amylolytic or diastatic enzymes (α and β amylases) that are naturally present in the flour catalyze the hydrolysis of damaged starch granules. The predominant sugar that is formed by such enzymatic reaction is maltose. Special strains of yeast with strong maltose-fermenting ability must, therefore, be selected to leaven such lean dough systems.

Generally, the maltose-utilizing enzymes within yeast are induced by the maltose present in the medium following the reduction of any glucose present to minimal concentrations (<0.1%). The maltose utilization by *Saccharomyces cerevisiae* strains require the induction of two proteins: α-D-glucosidase (maltase) and maltose permease. In the absence of glucose,

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ATTACHMENT D

YEAST TECHNOLOGY

Second Edition

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